Current Biology 19, 1616–1622, October 13, 2009 ©2009 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2009.07.073

Report

Notch Signaling Functions as a Cell-Fate Switch between the Endothelial and Hematopoietic Lineages

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Summary

Recent studies have begun to elucidate how the endothelial lineage is specified from the nascent mesoderm [1, 2]. However, the molecular mechanisms which regulate this process remain largely unknown. We hypothesized that Notch signaling might play an important role in specifying endothelial progenitors from the mesoderm, given that this pathway acts as a bipotential cell-fate switch on equipotent progenitor populations in other settings [3, 4]. We found that zebrafish embryos with decreased levels of Notch signaling exhibited a significant increase in the number of endothelial cells, whereas embryos with increased levels of Notch signaling displayed a reduced number of endothelial cells. Interestingly, there is a concomitant gain of endothelial cells and loss of erythrocytes in embryos with decreased Notch activity, without an effect on cell proliferation or apoptosis. Lineage-tracing analyses indicate that the ectopic endothelial cells in embryos with decreased Notch activity originate from mesodermal cells that normally produce erythrocyte progenitors. Taken together, our data suggest that Notch signaling negatively regulates the number of endothelial cells by limiting the number of endothelial progenitors within the mesoderm, probably functioning as a cell-fate switch between the endothelial and the hematopoietic lineages.

Results and Discussion

In order to determine the function of Notch signaling during the specification of the endothelial lineage, we first evaluated the effects of decreased Notch activity on endothelial cells at early stages of zebrafish development. *Tg(kdrl:EGFP)^{s843}* [5] embryos were treated with 100 μM DAPT (in 5% DMSO), a known chemical antagonist of Notch signaling [6], or 5% DMSO (vehicle alone) from 6 to 18 hr postfertilization (hpf), and the number of endothelial nuclei within a single transverse section of each embryo was quantified. At the tenth somite level, an average of 6.66 (s = 2.42) endothelial nuclei were present in embryos treated with DAPT (n = 44), in comparison to an average of 4.03 (s = 1.27) endothelial nuclei in control embryos treated with DMSO (n = 30), representing a 65% increase (analysis of variance [ANOVA], $p < 10^{-6}$) (Figure 1). Analysis at the fifth somite level yielded similar results (ANOVA, $p < 10^{-5}$; Figure S1, available online). The observed increase in the number of GFP-positive endothelial cells in DAPT-treated embryos does not appear to be a transgene-dependent phenomenon because similar increases were observed in other transgenic lines, such as *Tg(fli1a:nGFP)^{y7}* [7] and *Tg(kdrl:Ras-mCherry)^{s896}* [8], that have labeled endothelial cells (data not shown). In addition, a significant increase in the number of endothelial cells was also observed in homozygous *mindbomb* (*mib^{ta56b}*) mutant embryos, which lack an E3 ubiquitin ligase required for activation of Notch by Delta [9] (Figure S2).

We utilized fluorescence-activated cell sorting (FACS) to better quantify the effect of DAPT treatment on the entire endothelial population. For minimization of interference from the GFP expression in the pharyngeal endoderm of the *Tg(kdrl:EGFP)^{s843}* embryos [5], *casanova^{s4}* (*cas^{s4}*) mutant embryos [10] were used. Previous studies have shown that the *cas^{s4}* mutant embryos completely lack endoderm, with little effect on hematovascular development [10]. We found that DAPT-treated embryos contained 55% more GFP-positive endothelial cells in comparison to DMSO-treated embryos (Figure S3). Therefore, Notch signaling appears to negatively regulate the number of endothelial cells throughout the entire embryo. Consistent with our findings, multiple *notch* and *delta* genes are expressed in the zebrafish gastrula (Figure S4).

Previous studies have demonstrated that Notch signaling is required at multiple time points for regulation of various aspects of vascular development [11–15]. To define the time frame in which Notch signaling modulates the number of endothelial cells, we treated embryos with DAPT during distinct stages of development. Embryos treated from 6 to 10 hpf and from 14 to 18 hpf did not exhibit obvious changes in the number of endothelial cells. In contrast, embryos incubated with DAPT from 10 to 14 hpf displayed an increase in the number of endothelial cells similar to those continuously treated from 6 to 18 hpf (Figure S5 and data not shown). Interestingly, decreased Notch activity at this stage did not inhibit the differentiation of arterial endothelial cells (as determined by EphrinB2a expression), suggesting that the function of Notch signaling in limiting the number of the endothelial cells can be separated from the previously known function in promoting arterial endothelial cell fate.

For testing whether increased Notch activity might cause the opposite effect on the number of endothelial cells, *Tg(hsp70l:Gal4)^{kca4};Tg(UAS:myc-notch:intra)^{kca3}* [16] embryos were heat-shocked at shield stage (6 hpf), inducing the expression of the intracellular domain of Notch (NICD). Embryos expressing the NICD (detected by presence of the myc epitope) exhibited a significant decrease in the number

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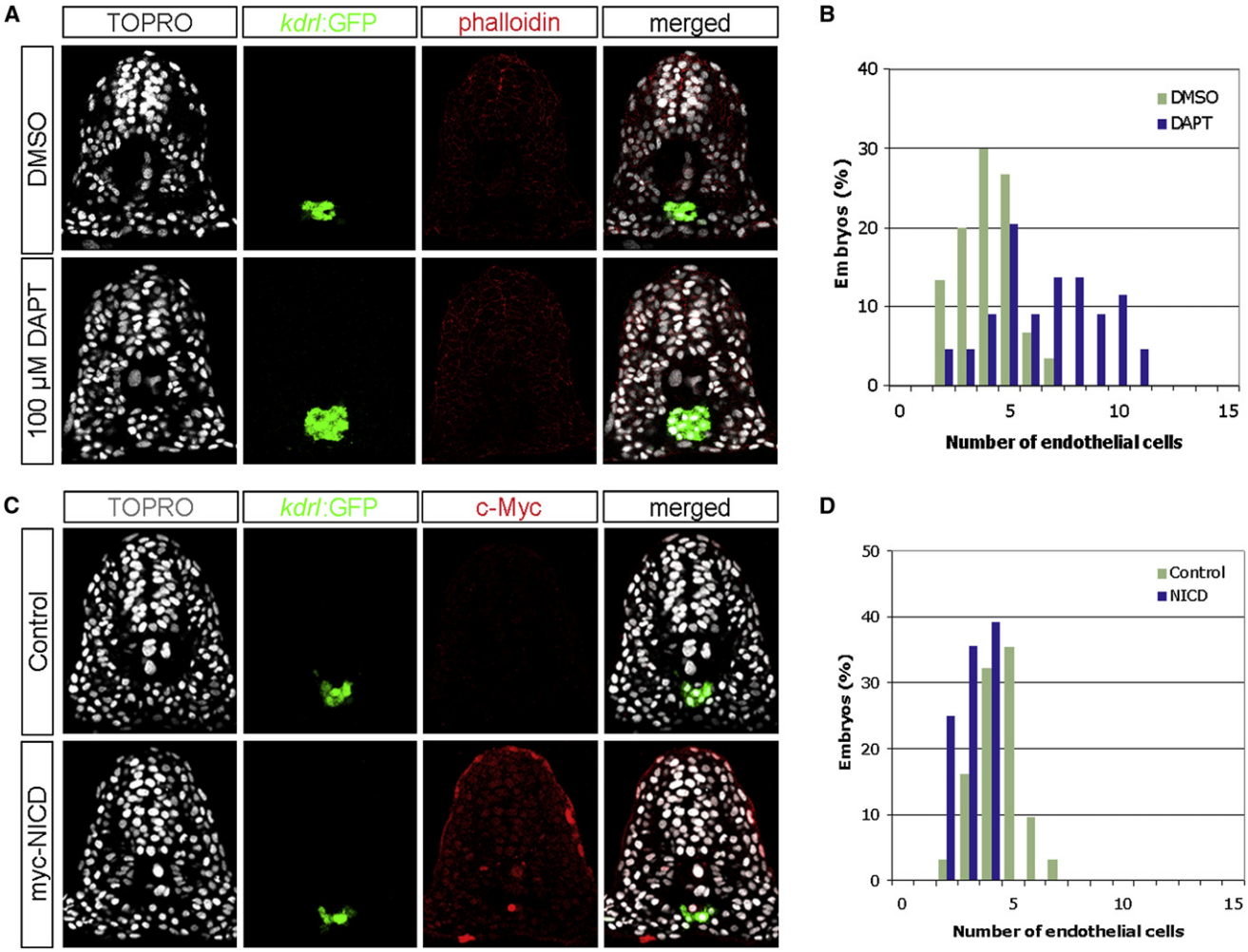

Figure 1. Notch Signaling Negatively Regulates the Number of Endothelial Cells during Zebrafish Development

(A) Transverse sections of 18 hpf embryos, DMSO- or DAPT-treated, visualized for TOPRO (white), *kdrl*:GFP (green), and phalloidin (red).

(B) Quantification of endothelial nuclei per focal plane in DMSO-treated (n = 30) or DAPT-treated (n = 44) embryos.

(C) Transverse sections of 18 hpf phenotypic wild-type siblings and *Tg(hsp70l:Gal4)*^{kca4}*;Tg(UAS:myc-NICD)*^{kca3} embryos visualized for TOPRO (white), *kdrl*:GFP (green), and c-Myc as a surrogate measure for NICD expression (red).

(D) Quantification of endothelial nuclei per focal plane in phenotypic wild-type (n = 31) and NICD-overexpressing (n = 28) embryos. Embryos treated with DAPT contained 6.66 (*s* = 2.42) endothelial nuclei per section, whereas those treated with DMSO had 4.03 (*s* = 1.27) endothelial nuclei (ANOVA, $p < 10^{-6}$). Conversely, embryos overexpressing the NICD contained 3.14 (*s* = 0.80) endothelial nuclei, in comparison to 4.42 (*s* = 1.09) nuclei per section in phenotypic wild-type siblings (ANOVA, $p < 10^{-5}$), suggesting that Notch signaling negatively regulates endothelial cell number.

of endothelial cells in comparison to the non-heat-shocked control siblings (Figure 1C). An average of 4.42 (*s* = 1.09) endothelial nuclei were present in the control embryos (n = 31), whereas an average of 3.14 (*s* = 0.80) endothelial nuclei were found in the embryos expressing NICD (n = 28), representing a 29% loss in endothelial cell number (ANOVA, $p < 10^{-5}$; Figure 1D). Therefore, increased Notch activity leads to a reduction in the number of endothelial cells. Taken together, our results show that Notch signaling negatively regulates the number of the endothelial cells during early zebrafish development.

To determine whether the observed gain of endothelial cells in embryos with decreased Notch activity was caused by changes in cell proliferation or cell death, we measured these rates in DAPT- and DMSO-treated embryos at 12, 14, and 16 hpf. Neither BrdU incorporation nor staining with the mitotic marker phospho-Histone3 showed any discernable differences in cell proliferation within the endothelial cells between DAPT- and DMSO-treated embryos (Figure S6). In addition, Acridine Orange and anti-Caspase3/8 staining did not show any differences in cell death between the two groups (data not shown), suggesting that these processes are not likely to be the major cause for the increased number of endothelial cells in embryos with decreased Notch activity.

We then speculated that the excess endothelial cells may have resulted from expansion of the endothelial progenitor population within the mesoderm, potentially at the expense of other related lineages. To test this hypothesis, we investigated the effects of modulated Notch signaling on the hematopoietic lineage, which develops in close spatiotemporal proximity to the endothelial lineage during embryogenesis [2, 17]. In DAPT-treated embryos, the expression levels of hematopoietic markers, such as *gata1* [18], *ikaros* [19], and *draculin* [20], were drastically decreased in comparison to those of

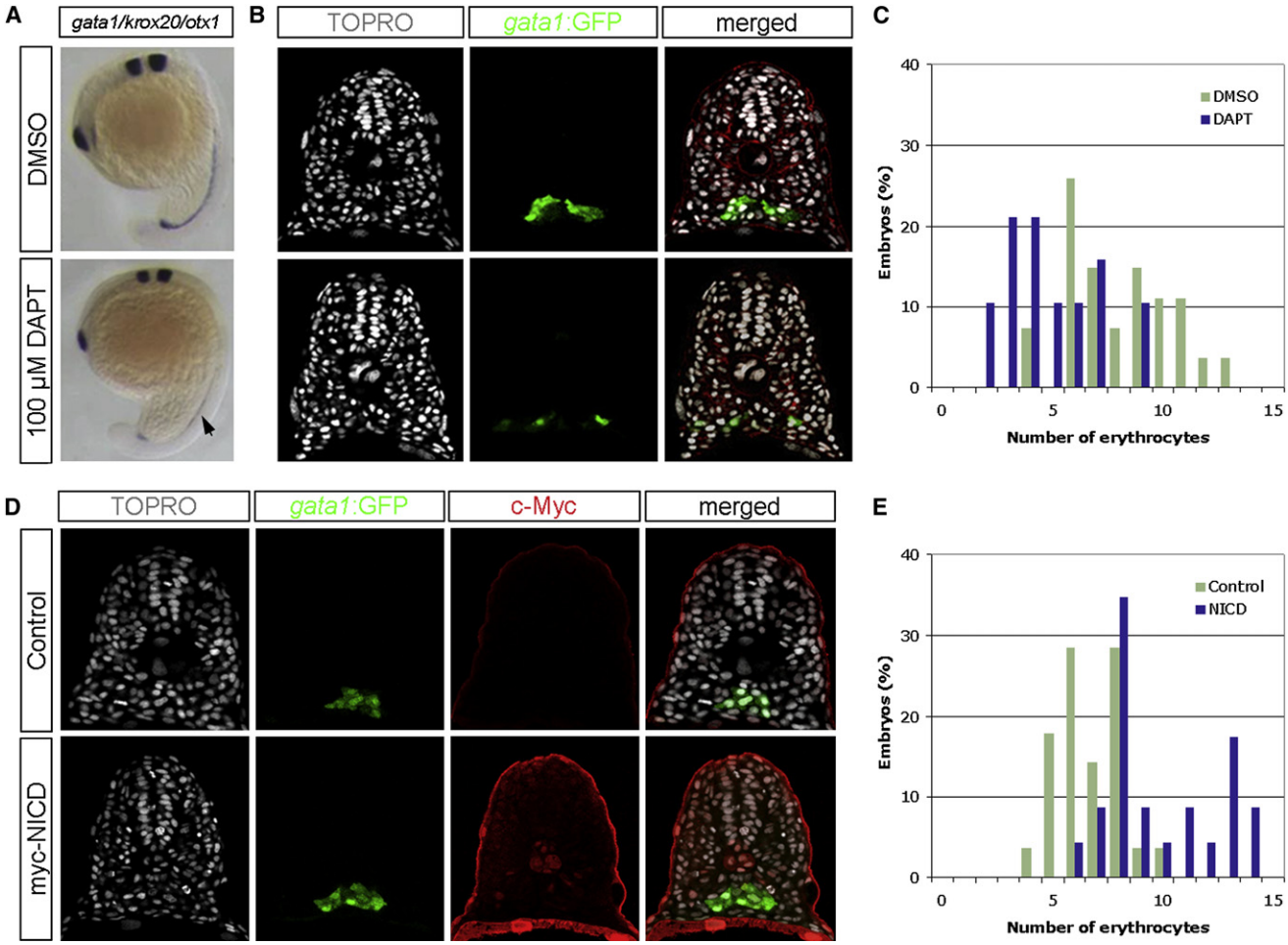

Figure 2. Notch Signaling Positively Regulates the Number of Hematopoietic Cells during Zebrafish Development

(A) Whole-mount RNA in situ hybridization showing the expression of *gata1* in 18 hpf DMSO-treated or DAPT-treated embryos. Two additional markers, *krox20* and *otx1*, which label the hindbrain and neuroectoderm, respectively, were used as positive controls. Notice the reduced expression of *gata1* in DAPT-treated embryos (black arrow).

(B) Transverse sections of 18 hpf DMSO- or DAPT-treated embryos visualized for TOPRO (white), *gata1*:GFP (green), and phalloidin (red).

(C) Quantification of the hematopoietic nuclei per focal plane in DMSO-treated (n = 27) or DAPT-treated (n = 19) embryos.

(D) Transverse sections of 18 hpf phenotypic wild-type siblings and *Tg(hsp70l:Gal4)^{kca4};Tg(UAS:myc-NICD)^{kca3}* embryos visualized for TOPRO (white), *gata1*:GFP (green), and c-Myc as a surrogate measure for NICD expression (red).

(E) Quantification of the hematopoietic nuclei per focal plane in phenotypic wild-type siblings (n = 28) and NICD-overexpressing embryos (n = 23). DAPT-treated embryos contained 4.89 (s = 2.15) erythrocyte nuclei, in comparison to 8.07 (s = 2.38) nuclei in DMSO-treated embryos (ANOVA, $p < 10^{-4}$). Conversely, embryos overexpressing the NICD contained 9.89 (s = 2.53) erythrocyte nuclei, in comparison to 6.72 (s = 1.43) nuclei per section in phenotypic wild-type siblings (ANOVA, $p < 10^{-6}$), indicating that Notch signaling positively modulates the number of hematopoietic cells.

DMSO-treated embryos (Figure 2A and data not shown). The reduced expression of these in situ markers appears to be specific to the hematopoietic lineage, because nonhematopoietic markers *krox20* [21] and *otx1* [22] (Figure 2A) show equivalent levels of expression.

To confirm that the reduced expression of hematopoietic lineage markers detected by in situ hybridization is the result of a decreased number of hematopoietic cells, we used *Tg(gata1:EGFP)^{la781}* [23] embryos to count the number of GFP-positive erythrocytes in DAPT- and DMSO-treated embryos. Given that we observed a drastic increase in the number of endothelial cells in the posterior half of the DAPT-treated embryos, where the majority of hematopoietic cells are erythrocytes, counting the number of *gata1*:GFP-positive cells would serve as a simple surrogate measure for identifying any discernable changes in the number of hematopoietic cells. In comparison to an average of 8.07 (s = 2.38) erythrocyte nuclei per section in DMSO-treated embryos (n = 27), embryos treated with DAPT (n = 19) had an average of 4.89 (s = 2.15) erythrocyte nuclei (Figure 2B), approximately a 39% decrease (ANOVA, $p < 10^{-4}$; Figure 2C). Conversely, embryos expressing the NICD exhibited a significant increase in the number of hematopoietic cells in comparison to control siblings (ANOVA, $p < 10^{-6}$; Figures 2D and 2E).

Unlike the hematopoietic lineage, no discernable changes in the number of somitic (detected with S58 and F59 antibodies [24]) or pronephric duct (*Tg(pax2a:GFP)^{e1}* [25]) cells were detected in embryos treated with DAPT, suggesting that the contribution of progenitors giving rise to other nonaxial mesodermal lineages is negligible (Figure S7 and data not shown).

To further characterize the effects of perturbed Notch signaling on the endothelial and hematopoietic lineages,

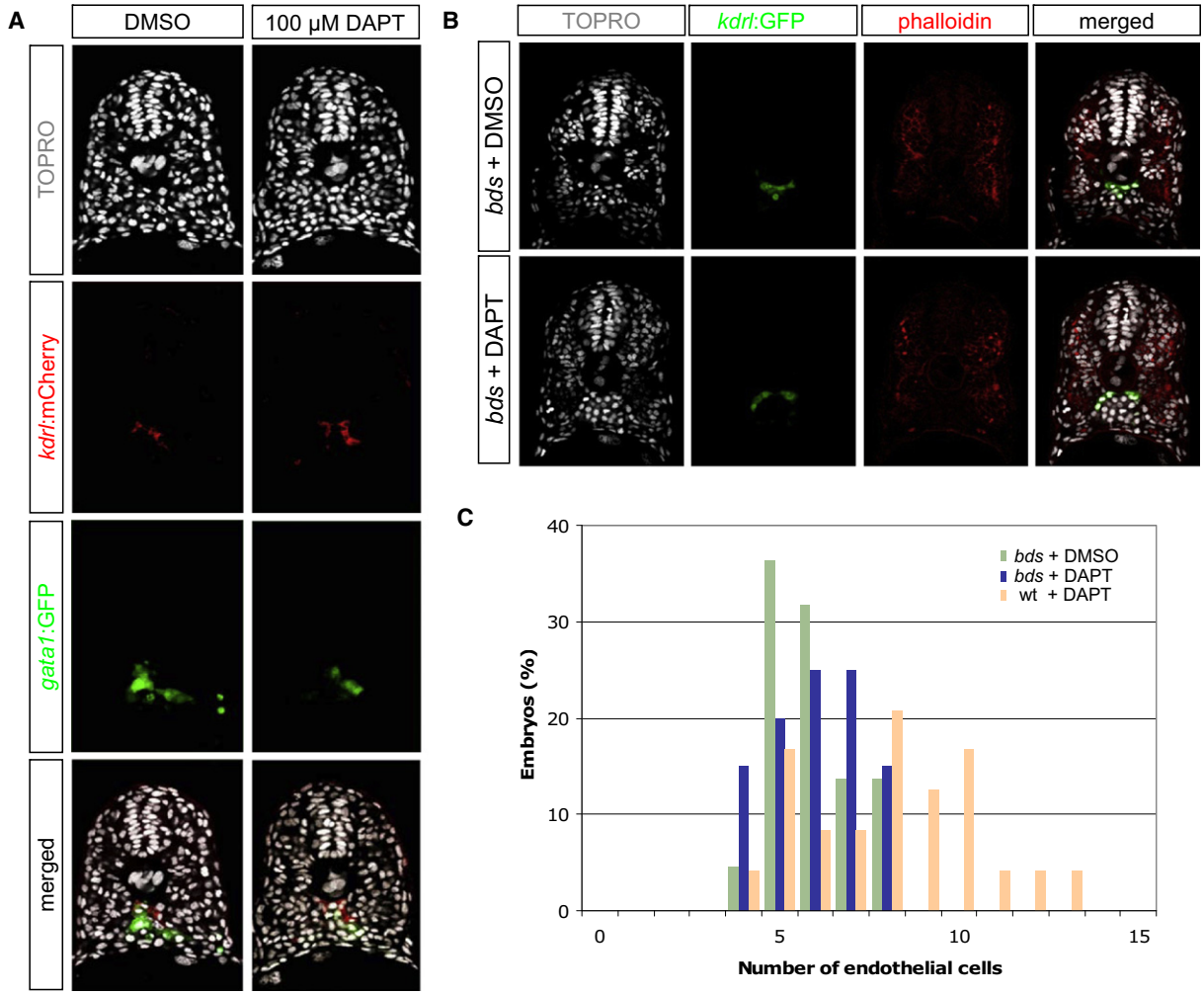

Figure 3. Reduced Notch Activity Causes an Increase of Endothelial Cells at the Expense of Hematopoietic Cells

(A) Transverse sections of 18 hpf DMSO- or DAPT-treated embryos, visualized for TOPRO (white), *kdrl*:mCherry (red), and *gata1*:GFP (green). DAPT-treated embryos exhibited a concomitant gain of endothelial cells and loss of hematopoietic cells.

(B) Transverse sections of 20 hpf DMSO- or DAPT-treated phenotypic *bloodless* (*bds*) mutant embryos, visualized for TOPRO (white), *kdrl*:GFP (green), and phalloidin (red).

(C) Quantification of the number of endothelial nuclei per focal plane in DMSO-treated *bds* (n = 22), DAPT-treated *bds* (n = 20), and DAPT-treated wild-type sibling (n = 24) embryos. DAPT treatment in embryos lacking primitive hematopoietic cells (*bds* + DAPT) did not result in a change in the number of endothelial cells, suggesting that the ectopic endothelial cells observed in DAPT-treated wild-type embryos might have originated from cells that normally produce hematopoietic cells.

we treated *Tg(kdrl:Ras-mCherry)*^{s896}*;Tg(gata1:EGFP)*^{la781} embryos with DAPT. Interestingly, the embryos exhibited a concomitant loss of erythrocytes and gain of endothelial cells (Figure 3A), suggesting that Notch signaling might regulate the number of endothelial and hematopoietic cells during the same developmental stages. For investigation of the possibility that there is a transformation of hematopoietic progenitors into endothelial progenitors at early stages in embryos with compromised Notch signaling, homozygous *bloodless* (*bds*) mutant embryos were treated with DAPT. The *bds* mutation obliterates all primitive hematopoietic cells via lineage-specific apoptosis, without affecting the endothelial lineage [26]. Thus, if hematopoietic progenitors within the mesoderm are transformed to endothelial progenitors as a result of decreased Notch activity, there should be no discernable change in the number of endothelial cells in the *bds* mutant embryos.

In DAPT-treated *bds* mutant embryos (n = 20), an average of 6.05 (*s* = 1.32) endothelial nuclei were present per section, and DMSO-treated *bds* mutant embryos (n = 22) and DAPT-treated phenotypic wild-type siblings (n = 24) contained an average of 5.95 (*s* = 1.13) and 8.04 (*s* = 2.36) endothelial nuclei, respectively (Figure 3B). Thus, we found no significant difference in the number of endothelial cells in *bds* mutant embryos treated with DAPT (ANOVA, $p < 0.80$; Figure 3C). These data suggest that decreased Notch activity causes a transformation of hematopoietic progenitors to endothelial progenitors and that Notch signaling might function as a cell-fate switch between the two lineages.

For further testing of the function of Notch signaling as a cell-fate switch between the endothelial and the hematopoietic lineages, lineage-tracing experiments were performed [2]. Single-cell-stage embryos were injected with DMNB-caged fluorescein-conjugated dextran. At shield stage,

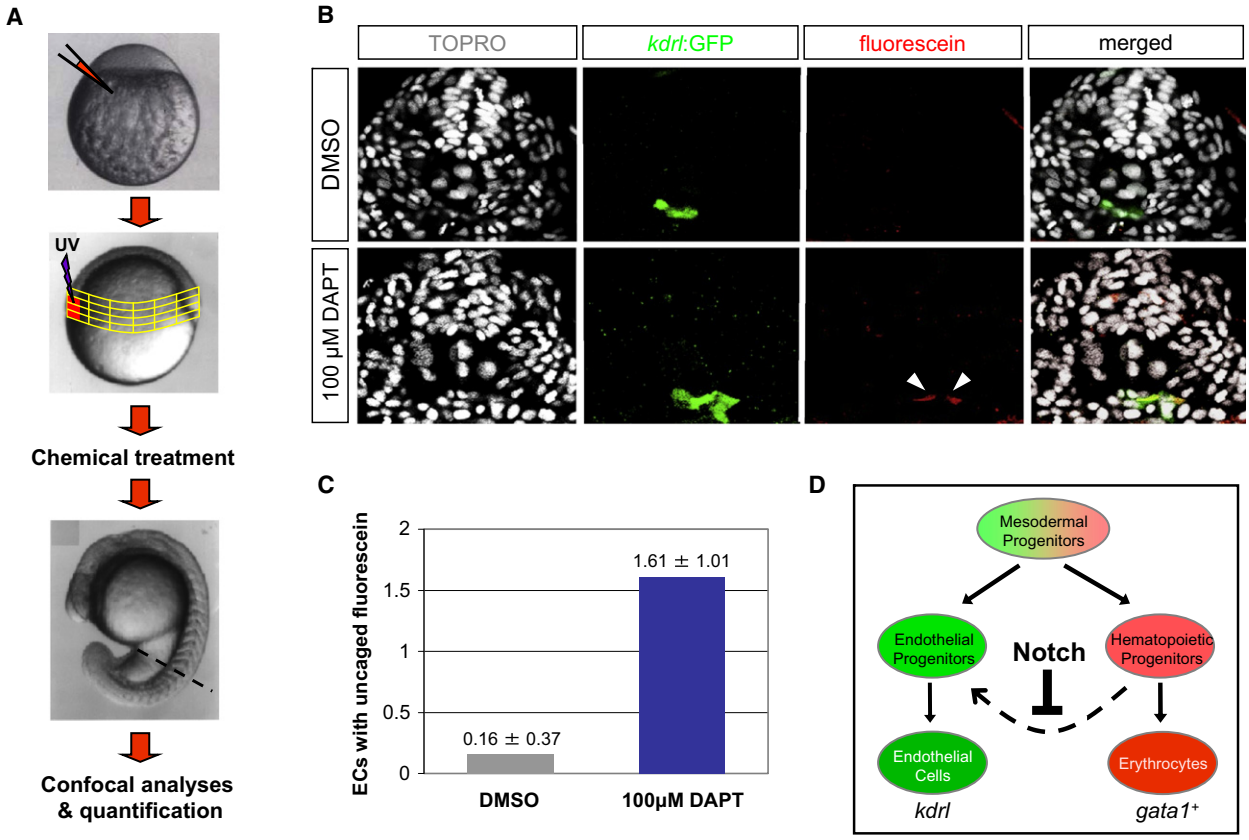

Figure 4. Notch Signaling Functions as a Cell-Fate Switch between the Endothelial and the Hematopoietic Lineages in the Nascent Mesoderm

(A) Experimental scheme of the lineage-tracing experiment. Single-cell-stage embryos were injected with DMNB-caged fluorescein-conjugated dextran. At shield stage, ten cells within the ventral margin, which predominantly gives rise to hematopoietic cells, were uncaged with UV irradiation. Subsequently, embryos were treated with either DMSO or DAPT until 18 hpf.

(B) Transverse sections of 18 hpf embryos treated with DMSO or DAPT after uncaging, visualized for TOPRO (white), *kdrl*:GFP (green), and uncaged fluorescein (red). White arrowheads point to endothelial cells that contain uncaged fluorescein in DAPT-treated embryos.

(C) Quantification of the number of endothelial nuclei with uncaged fluorescein per focal plane in embryos treated with DMSO (n = 18) or DAPT (n = 13). Embryos treated with DAPT contained 1.61 (*s* = 1.01) fluorescein-positive endothelial cells, in comparison to 0.16 (*s* = 0.37) in DMSO-treated embryos.

(D) Our proposed model: Notch signaling negatively regulates endothelial cell number by limiting the mesodermal cells that can differentiate into endothelial progenitors. Attenuated Notch activity causes mesodermal cells with hematopoietic potential to differentiate into endothelial progenitors, thus increasing the number of endothelial cells.

approximately ten cells within the ventral margin of the zebrafish gastrula, which predominantly generates the primitive hematopoietic lineage, were uncaged with UV irradiation. Subsequently, embryos were treated with either DAPT or DMSO (Figure 4A). If there is a direct transformation of hematopoietic progenitors to endothelial progenitors as a result of decreased Notch activity, the increased contribution to the endothelial lineage from the ventral margin should be detected. Because the cells from the ventral marginal zone rarely contribute to the endothelial lineage [2], an average of 0.16 (*s* = 0.37) endothelial cells per focal plane contained uncaged fluorescein in DMSO-treated embryos (n = 18) (Figures 4B and 4C). In contrast, in DAPT-treated embryos (n = 13), an average of 1.61 (*s* = 1.01) endothelial cells per focal plane showed colocalization with uncaged fluorescein (Figures 4B and 4C). These data support a model in which hematopoietic progenitors within the ventral margin of the zebrafish gastrula are transformed to endothelial progenitors upon the inhibition of Notch signaling, eventually leading to an increased number of endothelial cells (Figure 4D).

Considering the existence of common progenitors for the endothelial and hematopoietic lineages in zebrafish, it is tempting to speculate that Notch signaling might function as a bipotential cell-fate switch for the two lineages by regulating the hemangioblasts [2], resulting in descendents preferentially adopting an endothelial fate. However, the observed magnitude of increase in endothelial cells after inhibition of Notch signaling suggests that the contribution of such an event would be negligible, although we cannot exclude the possibility.

Conclusion

Our experiments provide evidence that Notch signaling negatively regulates the number of endothelial cells by modulating the specification of endothelial progenitors. This novel function of Notch signaling can be separated from a previously known function in promoting the differentiation of arterial endothelial cells during later stages of development. In addition, our data indicates that the effect of Notch signaling on endothelial cell number is the result of the direct transformation of hematopoietic progenitors into endothelial progenitors. Taken together, our results suggest that Notch signaling

functions as a cell-fate switch between the endothelial and the hematopoietic lineages within the nascent mesoderm.

Experimental Procedures

Zebrafish Husbandry, Morpholino Injection, and Chemical and Heat-Shock Treatments

Zebrafish (*Danio rerio*) embryos were obtained from the University of North Carolina, Chapel Hill (UNC-CH) Zebrafish Aquaculture Core Facility and raised as previously described [26]. The following mutant and transgenic lines were used: *bds*^{a75} [27], *cas*^{s4} [10], *mib*^{ta56b} [7], *Tg(gata1:EGFP)*^{la781} [23], *Tg(hsp70l:Gal4)*^{kca4} [16], *Tg(fli1a:nGFP)*^{y7} [8], *Tg(kdrl:EGFP)*^{s843} [5], *Tg(kdrl:Ras-mCherry)*^{s896} [9], *Tg(pax2a:GFP)*^{e1} [24], and *Tg(UAS:myc-notch:intra)*^{kca3} [16]. Homozygous *mib*^{ta56a} embryos were identified by PCR, and homozygous *bds*^{a75} embryos were identified on the basis of the lack of *ikaros* expression by RT-PCR.

Embryos were treated with 100 μM DAPT (CalBiochem) as previously described [6]. Embryos were treated from 6 to 18 hpf, unless noted otherwise, and subsequently fixed in 4% paraformaldehyde (PFA) overnight before processing. So that ectopic Notch signaling would be induced, embryos from crosses between *Tg(hsp70l:Gal4)*^{kca4}/+ and *Tg(UAS:myc-notch:intra)*^{kca3}/+;*Tg(kdrl:EGFP)*^{s843} individuals were raised at 28°C until shield stage (6 hpf) and subjected to treatment at 40°C for 30 min. Bright-field microscopy and *myoD* [28] in situ hybridization were used for verifying that the treated embryos exhibited no developmental delays in comparison to the control embryos (Figure S8).

Immunohistochemistry, In Situ Hybridization, and BrdU Incorporation

Immunohistochemistry and whole-mount in situ hybridization were performed as previously described [5]. The antibodies and riboprobes used can be found in the Supplemental Data. Polymerized actin was visualized by phalloidin (Molecular Probes) at 1:300, and nuclei were visualized with TOPRO (Molecular Probes) at 1:10,000. For immunohistochemistry, processed samples were mounted in Vectashield (Vector Laboratories) and the images were acquired with a Zeiss 510 Meta confocal microscope (Michael Hooker Microscopy Facility, UNC-CH) or a Zeiss LSM5 Pascal confocal microscope. For whole-mount in situ hybridization, embryos were mounted in benzylbenzoate:benzyl alcohol and documented with Zeiss Axiocam.

For BrdU incorporation, homozygous *Tg(kdrl:EGFP)*^{s843} embryos were treated with DAPT or DMSO from 10 to 18 hpf and also incubated in 10 mM BrdU in 5% DMSO from 14 to 16 hpf. At 18 hpf, embryos were fixed in 4% paraformaldehyde overnight and then incubated in rat monoclonal anti-BrdU followed by anti-rat Alexa Fluor 568.

Lineage Tracing

Lineage tracing was performed as previously described [2]. In brief, *Tg(kdrl:EGFP)*^{s843} embryos were injected with 4.6 nl of 0.2% DNMB-caged, biotinylated, lysine-fixable fluorescein dextran (Molecular Probes) in 0.2 M KCl and allowed to develop until shield stage (6 hpf). The cells located in the first three vegetal tiers of the ventral margin were activated with pulses from a 360 nm laser focused through a 20X objective. Embryos were then treated with DAPT or DMSO and fixed at 18 hpf. To detect activated fluorescein and distinguish it from GFP, we incubated embryos in biotinylated rabbit IgG FITC antibody followed by Alexa Fluor 568 Streptavidin (Molecular Probes).

ANOVA

The data were analyzed with one-way ANOVA for the effect of different treatments on the number of cells of interest. A p value smaller than 0.01 was regarded as significant.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and eight figures and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01624-8.

Acknowledgments

This work was initiated in the Stainier lab. The authors would like to thank the members of the Jin lab for helpful discussions and Cheol-Hee Kim and Hae-Chul Park for in situ probes. We also thank Victoria Bautch, Frank Conlon, Wiebke Herzog, Mark Majesky, Cam Patterson, and John Rawls for discussion and critical reading of the manuscript. This study was supported by an National Science Foundation predoctoral fellowship to K.M.V., the Biomedical Research Council of A*STAR, Singapore to Y.-J.J., the National Institutes of Health and Packard foundation to D.Y.R.S., and a Basil O'Connor Scholar Award from the March of Dimes Foundation, the National Heart, Lung and Blood Institute (HL090960), and the University of North Carolina at Chapel Hill Research Fund to S.-W. J.

Received: February 23, 2009
Revised: July 21, 2009
Accepted: July 23, 2009
Published online: September 10, 2009

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